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Prevalence of Lysogeny among Soil Bacteria and Presence of 16S rRNA and *trzN* Genes in Viral-Community DNA▽

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Bacteriophages are very abundant in the biosphere, and viral infection is believed to affect the activity and genetic diversity of bacterial communities in aquatic environments. Lysogenic conversion, for example, can improve host fitness and lead to phage-mediated horizontal gene transfer. However, little is known about lysogeny and transduction in the soil environment. In this study we employed atrazine-impregnated Bio-Sep beads (a cell immobilization matrix) to sample active microbiota from soils with prior pesticide exposure history. Once recovered from soil, the bead communities were induced with mitomycin C (MC), and viral and bacterial abundances were determined to evaluate the incidence of inducible prophage in soil bacteria. The inducible fraction calculated within bead communities was high (ca. 85%) relative to other studies in aquatic and sedimentary environments. Moreover, the bacterial genes encoding 16S rRNA and *trzN*, a chlorohydrolase gene responsible for dehalogenation of atrazine, were detected by PCR in the viral DNA fraction purified from MC-induced bead communities. A diverse collection of actinobacterial 16S rRNA gene sequences occurred within the viral DNA fraction of induced, water-equilibrated beads. Similar results were observed in induced atrazine-equilibrated beads, where 77% of the cloned sequences were derived from actinobacterial lineages. Heterogeneous 16S rRNA gene sequences consisting of fragments from two different taxa were detected in the clone libraries. The results suggest that lysogeny is a prevalent reproductive strategy among soil bacteriophages and that the potential for horizontal gene transfer via transduction is significant in soil microbial communities.

Viruses are extremely abundant in the biosphere. Over the past 15 to 20 years, considerable progress has been made in revealing the ecological role of viruses in aquatic ecosystems. Viral abundance, distribution, rates of production, diversity, and viral effects on microbial community structure and function have been topics of intense scientific interest (for comprehensive reviews see references 30 and 39).

In stark contrast to the burgeoning view of viral ecology in marine ecosystems are the limited data from terrestrial habitats. For example, the first reliable direct counts of soil viruses did not appear until 2003 (2, 36). Since that time viruses have been enumerated in other soil samples, but the abundance and distribution of viruses in soils (depth, soil type, land management, temporal variability, etc.) remain an open question. Initial studies indicate that soil viral communities are more abundant and diverse than their aquatic counterparts (35). Across six soils examined with different land use patterns, viral abundance ranged from 9×10^8 to 4×10^9 viruses gram (dry weight) (gdw) soil^{-1}. Viral abundance was significantly correlated with soil moisture, with forested wetland soil having the highest viral abundance. Interestingly, viral abundance and the ratio of viral to bacterial abundance (VBR) were correlated with differences in land use. Moist and organic-matter-rich forested soils showed high ($>10^9$ virus gdw^{-1}) viral abundance and modest VBR values of around 10. In contrast, dry and organic-matter-poor agricultural soils showed lower viral abundance ($<10^9$ to 10^8 virus gdw^{-1}) and astoundingly high VBR values of around 3,000.

The prevalence of lysogeny in prokaryotic communities cannot be measured directly but is typically estimated by enumerating viral and bacterial abundance in environmental samples exposed to various inducing agents such as mitomycin C (MC) or UV radiation and comparing these values to control samples, often with highly variable results. For example, the fraction of bacterioplankton-containing inducible prophages in estuarine and coastal seawater ranged from a low of 0.07% (31) to a high of 42% (13). Approximately 41% of cultivable heterotrophic marine bacteria contained MC-inducible prophage (14); however, Wilcox and Fuhrman (33) found no evidence for lysogenic bacteriophage production in bacterial communities from coastal seawater. In Lake Superior, low levels of lysogeny ranging from 0.1 to 7% were reported (25), while in ice-covered Antarctic lakes values as high as 89.5% have been observed (16). The incidence of lysogeny in marine environ-

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ments also appears to exhibit seasonal patterns (5, 37). Fewer studies have been done in marine sediments, but the available data suggest that the incidence of lysogeny is quite low, with only 2% of the bacterial community containing MC-inducible lysogens (18).

More than 20 years ago, Stewart and Levin (24), and later Marsh and Wellington (17), suggested that the soil environment likely selects for lysogenic reproduction in soil phages. The heterogeneous nature of soil leads to spatial separation and a patchy distribution of host bacteria, frequent nutrient limitations, and extraordinary diversity but highly even communities and low host growth rates, all factors which should favor the increased survival of temperate phages. In two previous studies, we examined the frequency of lysogeny among cultivable soil bacteria and found that approximately 30% contained inducible prophage (35a), while cultivation-independent assessments ranged from 22 to 68% in soils from Delaware and 4 to 20% in Antarctic soil, suggesting that lysogeny may be more prevalent in soil microbial communities than in their marine counterparts (34).

Due to the random nature with which generalized transducing phage accidentally package host DNA during phage assembly, it is reasonable to expect all host genes to be transferred with equal frequency. Even host 16S rRNA genes have been detected in broad-host-range bacteriophage (4) and viruses sampled from wastewater treatment systems (23). In the cases of 16S rRNA genes detected within environmental viral samples, the source of these genes is believed to be generalized transducing phage.

Thus, we hypothesized that bacteriophages as a potential agent of gene transfer in soil could also carry pesticide catabolic and 16S rRNA genes. In this study we demonstrate that a high percentage of soil bacteria sampled with Bio-Sep beads carry prophage that can be induced by MC. We also show that viral preparations from induced Bio-Sep bead communities contain 16S rRNA and *trzN*, a gene that encodes atrazine chlorohydrolase. Actinobacteria were the predominant bacterial host group detected within 16S rRNA gene clone libraries prepared from the viral DNA fraction. Critical examination of some of the sequences revealed hybrid 16S rRNA structures indicative of interfamily horizontal transfer and recombination of 16S rRNA genes.

MATERIALS AND METHODS

Sampling microbiota with Bio-Sep beads. In an unrelated study that focused on the phylogenetic diversity of atrazine-degrading soil bacteria, Bio-Sep beads baited with atrazine (20 mg/kg) were used to sample indigenous soil bacteria. The beads (2- to 3-mm diameter) were originally developed as a cell immobilization matrix and consist of a composite of 25% aramid polymer (Nomex) and 75% powdered activated carbon (7, 32). These beads are highly porous and possess a high sorptive capacity. Sterile beads were impregnated with atrazine or equilibrated with sterile, deionized water and placed in nylon mesh bags prior to burial in soil for 4 to 6 weeks. The beads provide an ideal porous matrix with a high surface area that mimics the soil environment. Thus, atrazine-baited beads provided a matrix for the in situ enrichment of atrazine-degrading bacteria. The beads are readily recoverable from soil and can be used for cultivation studies or can be directly extracted for nucleic acid and lipid biomarkers for microbial community analyses. Both types of beads were deployed at two study sites (1B and 3A) in Ohio and one site in western Tennessee. All three sites were used for corn production and had long histories (>5 years) of atrazine treatment. After recovery from the field, all of the beads contained viable microbial biomass as determined by cultivation on agar plates and by fluorescent in situ hybridization using universal eubacterial probe EUB338 (data not shown). In the present study, subsamples of the field-incubated beads were used in MC induction experiments to determine the presence of lysogenic bacteria in the bead communities and to study the resulting viruses that were produced.

Induction of bead samples. In preliminary induction experiments with phosphate buffer, significant increases in viral abundance in MC-induced or control samples were not observed. Thus, induction assays were carried out in 0.1× Trypticase soy broth (TSB; 0.5 ml) in microcentrifuge tubes containing individual beads and treated with MC (1 $\mu g\ ml^{-1}$) or left unaltered as uninduced controls. Each assay mixture was incubated statically at room temperature in the dark for 18 h prior to extraction and purification of bacteria and viruses.

Viral and bacterial extraction and purification of viral DNA. Viruses were extracted from triplicate or quadruplicate beads from each location as described by Williamson et al. (36), and viral DNA was purified as described by Sander and Schmieger (23) and Beumer and Robinson (4) with some modifications. Briefly, MC-induced and uninduced control beads (one bead per tube in quadruplicate) were crushed with a sterile glass rod, and 700 μl extraction buffer (containing, per liter, 10 g potassium citrate, 1.44 g $Na_2HPO_4 \cdot 7H_2O$, 0.24 g KH_2PO_4, pH 7) was added. All tubes were vortexed at the highest speed for 20 min and centrifuged at 12,000 × *g* to sediment bead particles along with the adhering bacteria. Supernatants were passed through 0.22-μm syringe filters to remove any small bead particles and bacteria. To minimize the effects of any nonencapsulated DNA on the enumeration of phages and subsequent PCR analyses, RNase-free DNase (Fisher Scientific) was added to the phage extracts (2 U $ml\ extract^{-1}$) and the samples were incubated at room temperature for 25 min followed by addition of 0.4 M EDTA (6 $\mu l\ ml^{-1}$) to stop further DNase action. In separate control experiments bacterial genomic DNA (400 ng) from an atrazine-degrading bacterium, *Pseudomonas* strain ADP, containing the catabolic genes *atzABCDEF*, was added to some viral extracts prior to addition of DNase. After DNase digestion, samples were taken for PCR using *atzB*-specific primers. In all cases, *atzB* was undetectable in digested samples but was readily amplified from undigested control samples, suggesting that the DNase digestion effectively removed any DNA that may have contaminated the samples. After extraction and treatment with DNase, phage extracts were concentrated and washed in phosphate-buffered saline using Amicon spin filters (95-kDa molecular mass cutoff). The recovered phage concentrates (250 μl) were then heated to 90°C for 15 min and cooled, and DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 7.3) and 2.5 volumes of absolute ethanol. Bacteria from the bead samples were extracted using Nycodenz (Axis-Shield PoC AS, Oslo, Norway) density gradients using the high-speed centrifugation method (3, 8). Extraction buffer was added to the bead sediments (1.2 ml) and vortexed horizontally for 15 min. The entire samples were then layered upon 500 μl of Nycodenz (Axis-Shield PoC AS, Oslo, Norway) and centrifuged at 10,000 × *g* for 40 min. Bacteria were harvested from the upper layer. The resulting bacterial suspensions were washed once in sterile saline to remove residual Nycodenz, and the bacteria were resuspended in sterile phosphate-buffered saline prior to enumeration.

EFM. Viruses were enumerated as described by Williamson et al. (36) with minor modifications. Purified phage extracts were passed through 0.02-μm Anodisc filters and stained with Sybergold DNA stain for 20 min. Filters were analyzed by epifluorescence microscopy (EFM) using a Nikon Eclipse E600 microscope (Nikon Instrument Group, Melville, NY) with a fluorescein isothiocyanate excitation filter set. Twelve fields per sample were digitally photographed at a magnification of ×1,000 with a Q-Imaging Retiga Exi charge-coupled device camera, and viruses were counted with the aid of IPlab software (version 3.9; Scanalytics, BD Biosciences, Rockville, MD). Viruses were discriminated from bacteria or other fluorescing particles based on pixel dimension. Virus counts were calculated based on the grand mean of four replicate filters of beads from each location.

Bacteria. Bacterial suspensions were filtered and stained as described by Williamson et al. (35) except that 0.22-μm Durapore membrane filters were used instead of black polycarbonate filters. Bacteria were enumerated using EFM by counting the cells in 12 fields per filter at a magnification of ×1,000 using the same system described above. Average bacterial counts were calculated based on a grand mean of three or four replicate filters for beads from each location.

Transmission electron microscopy. Purified viral extracts from MC-induced Bio-Sep bead communities were examined by transmission electron microscopy to confirm the presence of phages and the absence of any obvious bacterial contamination as previously reported for soils, except that ultracentrifugation in $CsCl_2$ gradients was omitted (35). Phage particles were sedimented onto Formvar-coated 400-mesh copper grids by ultracentrifugation at 30,000 × *g* for 1 h. Grids were gently blotted with filter paper and stained with saturated uranyl acetate (aqueous) for 1 min. Excess stain was wicked away with filter paper, and grids were air dried before examination in a Zeiss CEM902 transmission electron microscope (acceleration voltage, 80 kV).

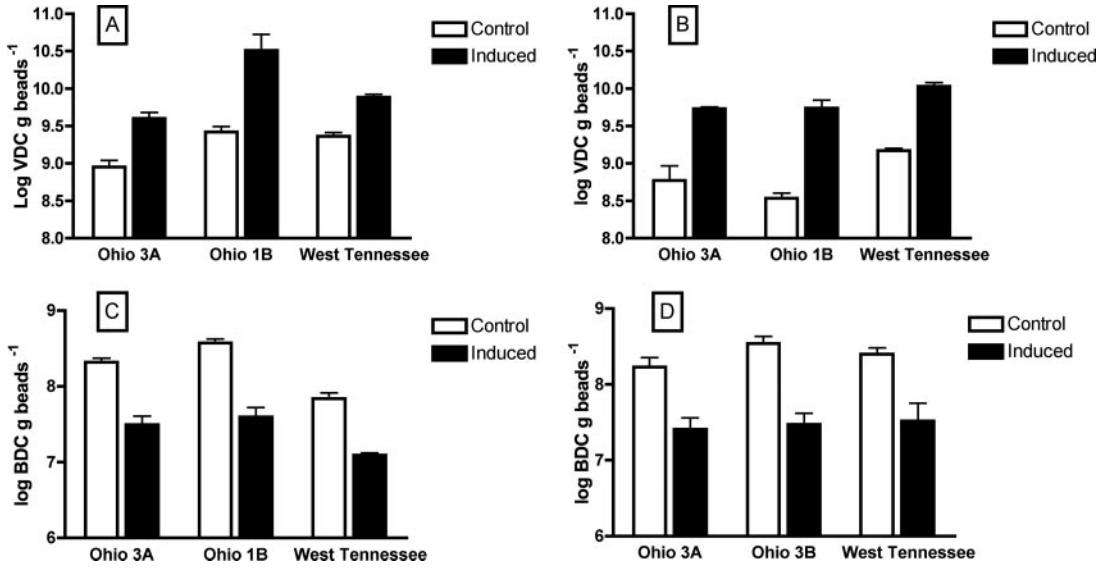

FIG. 1. Changes in viral and bacterial abundance after induction with MC in Bio-Sep beads that were previously equilibrated with atrazine (A and C) or water (B and D) and buried at the study sites for 4 to 6 weeks prior to induction. BDC, bacterial direct count; VDC, viral direct count.

Calculations of the IF of the bacterial population and induced burst size. The number of induced prophage was calculated as $VDC_I - VDC_C$, where VDC_I is the number of viruses enumerated in the induced sample and VDC_C is the number of viruses in the control sample. The MC-inducible fraction (IF) of the bacterial population was determined by the equation $IF = [(VDC_I - VDC_C)/B_Z]/BDC_C \times 100$, where BDC_C is the number of bacteria enumerated in the control sample and B_Z is the induced burst size (37). Induced burst size was calculated using the equation $B_Z = (VDC_I - VDC_C)/(BDC_C - BDC_I)$, where BDC_I is the number of bacteria enumerated in the induced sample.

PCR amplification of 16S rRNA genes and *trzN*. The DNA concentration in the viral extracts was determined with fluorometry, and 1 to 5 ng DNA was used as template in PCRs. PCR was performed using universal 16S rRNA primers, 11f (5′-GTTTGATCCTGGCTCAG-3′) and 1327R (5′CTAGCGATTCCGACTTCA-3′) (15). For *trzN*, forward (5′-GCGACGGGAAGTTCGGTC-3′) and reverse (5′-CGAGCGTCATCGATGACCT-3′) primers were designed to amplify a 200-bp fragment of all known *trzN* sequences. Each 50-μl reaction mixture contained 1× PCR buffer B, 2.5 U of *Taq* polymerase (Promega, Madison, WI), primers (0.4 μM), deoxynucleoside triphosphates (0.2 mM), and 2.5 mM $MgCl_2$. PCR conditions consisted of an initial denaturation step at 95°C for 2 min followed by 35 cycles of denaturation (60 s at 95°C), annealing (60 s at 58°C), and extension (110 s at 72°C) with a final extension step at 72°C for 5 min. After purification, 16S rRNA gene amplicons were cloned, sequenced, and analyzed as described below. The PCR products of *trzN* reactions were sequenced directly without cloning.

Preparation of 16S rRNA clone library and phylogenetic analysis. Amplification products were verified by gel electrophoresis and purified using the Wizard PCR cleanup kit (Promega, Madison, WI). Purified fragments were ligated to the pGEM-T vector (Promega), transformed into *Escherichia coli* strain JM109 competent cells, and plated on LB medium (with 50 μg ml^{-1} ampicillin) following the manufacturer's instructions. After overnight incubation, transformants were randomly selected and plasmids were extracted using the Wizard *Plus* Miniprep DNA purification system (Promega). Plasmid insert DNA was bidirectionally sequenced using M13 forward/reverse primers and the Big Dye cycle sequencing kit (Applied Biosystems, Foster City, CA). Approximately 400 ng of DNA from each cycle sequencing reaction mixture was sent to the University of Tennessee DNA sequencing facility. Here, products were purified using Autoseq G-50 columns (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and then separated and analyzed with an automated capillary DNA sequencer (ABI model 377XL; Applied Biosystems). Retrieved sequences were compared with available 16S rRNA gene sequences in GenBank using BLAST (1). Sequences were aligned with 16S rRNA sequences from a broad phylogenetic range of bacteria by using Clustal W (26).

Screening for chimeras. Potential chimeras in 16S rRNA gene sequences were evaluated using CCODE (11), Bellerophon (12), and RDP's CHECK CHIMERA (6) programs.

RESULTS

Bio-Sep beads as a culture-independent induction matrix. Bio-Sep beads have many environmental applications, e.g., as a cell immobilization matrix for the treatment of sulfidic wastes associated with petroleum refining (7) and as traps to sample active bacterial communities in contaminated groundwater aquifers (22). In a separate study we investigated the potential use of Bio-Sep beads baited with atrazine as a tool to recruit and sample indigenous soil bacteria able to metabolize this widely used herbicide. The goal was to enrich for slowly growing atrazine-degrading bacteria in situ that might otherwise be missed by traditional laboratory cultivation techniques. The beads were recovered from soil after 4 to 6 weeks of incubation and found to be a very useful matrix to entrap soil microbiota. Microscopic direct counts of bacteria extracted from beads were at least 10-fold higher than those of bacteria extracted directly from soil (data not shown). Subsamples of these beads were used in the induction experiments described below.

Prophage induction with MC. Beads incubated in two Ohio soils and one west Tennessee site were processed to determine the fraction of the community possessing prophage that could be induced by exposure to MC. No other chemical agents or environmental stressors such as UV light exposure were tested; thus, these measurements should be considered conservative estimates of the lysogenic fraction within the bead communities, as some lysogenic bacteria are not inducible with MC (30). After 15 to 18 h of exposure to MC, viral abundance significantly increased (ca. 1 to 2 orders of magnitude) and bacterial abundance decreased relative to uninduced control samples in both the water- and atrazine-equilibrated bead communities, and the difference was attributed to phage production from lysogenic bacteria (Fig. 1). Using the previously published equations of Williamson et al. (37), we estimated that 78 to 89% of the bacteria in the beads were lysogenic (Table 1). The mean number of total viruses (free viruses and induced prophage) enumerated for all MC-treated samples ranged from

TABLE 1. Burst sizes and IFs from induced Bio-Sep bead communities

Sample	Equilibration	Burst size	IF
Ohio site 1	Atrazine	9 ± 1	86 ± 6
	Water	36 ± 24	78 ± 23
Ohio site 2	Atrazine	121 ± 96	87 ± 10
	Water	19 ± 10	89 ± 8
Tennessee	Atrazine	102 ± 41	82 ± 6
	Water	58 ± 28	81 ± 18

9.6 ± 0.13 to 10.5 ± 0.43 (log virus direct count ± standard deviation g beads^{-1}), and the estimated average burst size for induced bacteria ranged from 14 ± 6 to 87 ± 32 viral particles host cell^{-1}. In addition to EFM, the viral extracts from the MC-induced samples were examined by transmission electron microscopy. Tailed phages belonging to the *Siphoviridae* and *Myoviridae* were visible (Fig. 2).

Detection of bacterial genes in soil phages. Viruses produced after exposure of microbial communities to MC were extracted and purified following previously published procedures with modifications (4, 23). DNA was then extracted from the virus fraction and used as a template for amplification of *atzA* and *trzN*, chlorohydrolase genes leading to the dehalogenation of atrazine, and 16S rRNA genes, with great care taken to avoid contamination by bacterial DNA. The *trzN* gene was readily detected in viral DNA collected from water- and atrazine-equilibrated beads; however, amplification of *atzA* was negative in all samples (Fig. 3). The 16S rRNA gene products of 1,300 bp were readily amplified from all samples except the appropriate negative controls and the filtrate from Amicon filters that were used to concentrate viral samples and remove any undigested, nonencapsulated DNA from the viral extracts.

Clone libraries of 16S rRNA genes constructed from the viral DNA fraction of water- and atrazine-equilibrated beads showed differences in their composition although an insufficient number of clones were analyzed to accurately assess diversity (Table 2). About 25 to 35 randomly picked clones from 16S rRNA gene libraries prepared from the water-equilibrated beads showed considerable genus-level diversity in *Actinobacteria*, but only 12 and 8% of the cloned sequences were closely related to *Alphaproteobacteria* and *Betaproteobacteria*, respectively (Table 2). No other taxa were detected

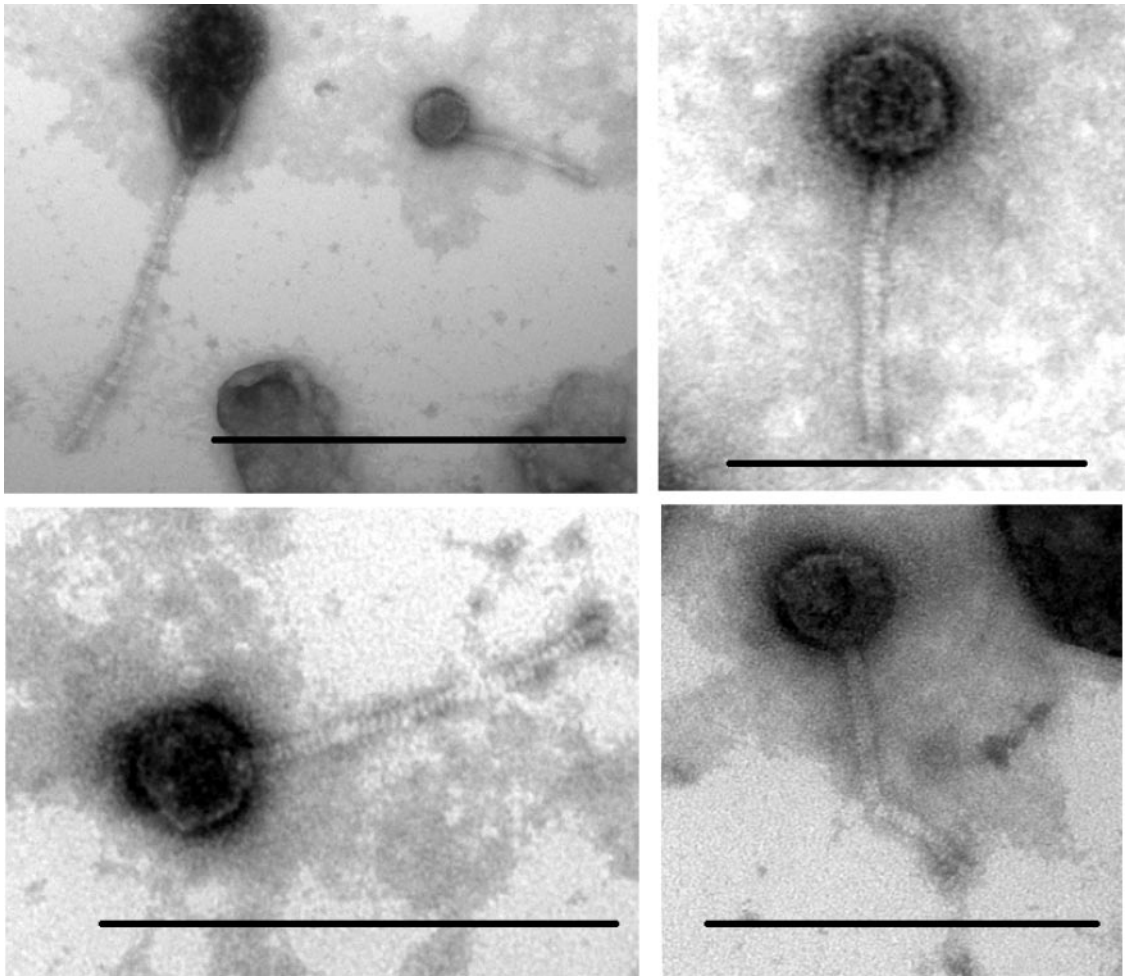

FIG. 2. Electron micrographs of viral samples extracted from induced beads. Bars, 200 nm (all panels).

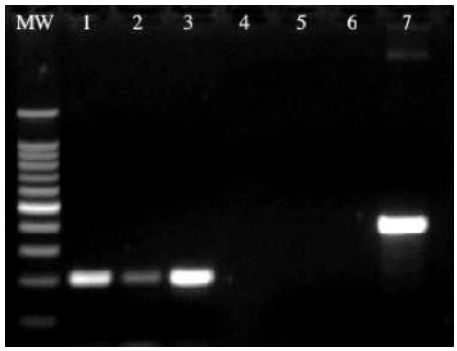

FIG. 3. Agarose gel (1.75%) electrophoresis of PCR products amplified with *trzN* primer sets from induced viral DNA (lane 1), uninduced viral DNA (lane 2), and DNA extracted from *Arthrobacter aurescens* (control for *trzN*) (lane 3). Lanes 5 and 6 contained PCR products from the same viral samples amplified with *atzA* primer sets. Lane 7 contains PCR product amplified with *atzA* primers from *Pseudomonas* sp. strain ADP. Lane 4, blank; MW, molecular weight markers.

among the clones analyzed. The clone library prepared from atrazine-equilibrated beads yielded approximately the same percentage of actinobacterial sequences, albeit fewer genera within this division were represented relative to water-equilibrated bead communities (Table 2). Also, in contrast to the water-equilibrated beads, more novel 16S rRNA gene sequences (i.e., those with lower similarity with sequences from cultivated bacteria in the GenBank database) were detected. Further examination of these sequences revealed family-level heterogeneity of 16S rRNA sequences.

Mosaic 16S rRNA genes. Thorough examination of sequences retrieved from clones (Table 2) revealed unusual heterogeneity in several of the recovered sequences. Sequences AIB-1, -11, and -21 were found to be identical and were 6 to 7 nucleotides longer in helix 43 (*E. coli* positions 1118 to 1155) (21) than their closest cultured GenBank homolog *Marmoricola* sp. (family *Nocardioidaceae*) with an expectation value of 0.0 (bitscore = 1,915) over the 1,300-bp sequence. Further BLAST analysis of only helix 43 in region V7 (nucleotides 1127 to 1189, *E. coli* numbering) for these clones returned a different top hit within GenBank, *Microbispora* (family *Streptosporangiaceae*), which yielded lower expectation values (2×10^{-26}) than those obtained for the sequences belonging to members of the family *Nocardioidaceae* (E value, 6×10^{-20}). Closer examination using Clustal W alignment of the V7 region for representative species of these two families revealed that 143 species of *Streptosporangiaceae* possessed sequences that were 5 to 7 nucleotides longer than 149 of 159 species examined within the *Nocardioidaceae* (Fig. 4). Furthermore, all of the *Streptosporangiaceae* sequences examined contained a thymine residue ("T") at position 1116 just 20 nucleotides upstream of the observed insertion while all of the *Nocardioidaceae* sequences possessed a cytosine residue at this position. Although the AIB-1 clone showed the highest bitscore and lowest E value with the members of *Streptosporangiaceae* within the V7 region, this signature nucleotide at position 1116 was a cytosine as in the *Nocardioidaceae* (Fig. 4) rather than a thymine. The close proximity of the observed inserted sequence (beginning at nucleotide 1138) to the conserved nucleotide residue at position 1116 indicates that the insertion was acquired by lateral gene transfer. Screening the sequences for possible chimeras using chimera check programs excluded the possibility that the observed differences in the V7 region were the result of PCR artifacts (11, 12).

DISCUSSION

Bio-Sep beads as matrix to sample active soil microbiota for lysogen induction assays. In this study, we sampled soil microbiota from several locations using porous beads and examined the communities that colonized the beads for production of temperate bacteriophage upon induction with MC. In other studies Bio-Sep beads have been shown to be a good matrix for colonization by bacteria (10, 22). Lipid and quinone profiles as well as live-dead microscopic assays from these studies suggest that the colonized Bio-Sep bead communities are more active and less stressed than the total bacterial community sampled from bulk soil. The induction assay data collected in this study were less variable than those from similar experiments that we have performed with whole soils (34). Thus, the beads appear to be an excellent alternative to intact natural samples to examine phage-host interactions.

The IF values (80 to 89%) calculated in this study from the bacterial populations in beads incubated in different soils were substantially greater than values reported for marine sediments (18) or in whole soils from Delaware and Antarctica (34). There has been only one other report of the lysogenic fraction or IF exceeding 80%, and this was reported for bacterial communities in Antarctic lakes (16). However, in this

TABLE 2. Sequencing results of 16S rRNA gene clones prepared from MC-induced Bio-Sep bead communities using the viral DNA fraction as template for PCR[a]

Bead type	Phylogenetic affiliation (GenBank accession no.)	No. of clones	% Similarity
Water equilibrated	*Kribella* sp. (AY253863)	1	98
	Rhodococcus sp. (AY785730)	2	99
	Microlunatus (AB245389)	3	98
	Arthrobacter sp. (AB089841)	5	98
	Frankia sp. (AF063641)	1	97
	Amycolatopsis sp. (AY129769)	1	99
	Saccharothrix sp. (AF328678)	2	98
	Microbacterium sp. (AY439232)	2	99
	Nocardioides sp. (AJ549286)	3	98
	Chelatococcus sp. (AJ871433)	1	97
	Methylobacterium (AB220099)	2	99
	Azohydromonas sp. (AB201626)	2	98
Atrazine equilibrated	***Marmoricola* sp. (EF466120)**[b]	**3**	**92**
	Uncultured actinobacterium (AY922063)	3	92
	Virgosporangium sp. (AB006169)	3	92
	Nocardioides sp. (AJ549286)	4	97
	Marmoricola sp. (AF408953)	4	95
	***Arthrobacter* sp. (AB098573)**[c]	**3**	**94**
	Arthrobacter sp. (AY572478)	2	97
	Arthrobacter sp. (AY572478)	3	98

[a] Boldface designates the clones having hybrid sequences.
[b] Corresponds to clones AIB-1, -11, and -21.
[c] Corresponds to clones AIB-4, -14, and -24.

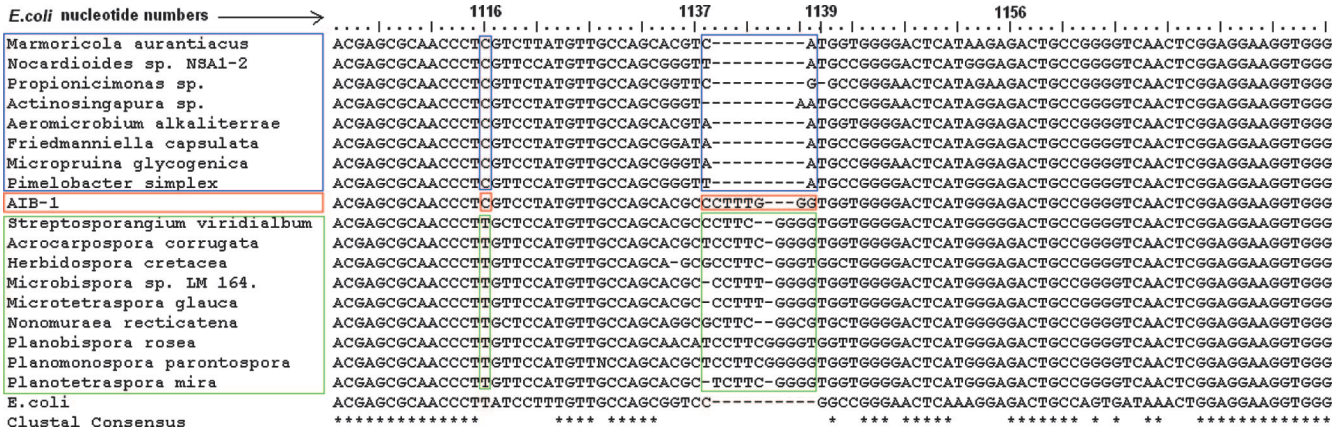

FIG. 4. Sequence alignment results for the members of the *Streptosporangiaceae* (species within the green box) and *Nocardioidaceae* (species within the blue box) in the V7 region of the 16S rRNA gene.

study, the higher value for IF was based only on viral production. When corresponding decreases in bacterial abundance were considered (as in the present study), the estimated IF values ranged from 2 to 62.5% and were more consistent with IF values reported for other aquatic environments. Two factors may account for the unusually high IF values that we observed in Bio-Sep bead communities. First, in the absence of nutrients (i.e., buffer only), an induction response was not observed and thus subsequent experiments were performed in dilute TSB. Addition of these nutrients may have stimulated bacterial growth and increased subsequent lytic phage production of the most rapidly growing bacterial population(s) within the beads. In the IF calculation, viral direct counts from lytic phage production would be subtracted from the total as the control samples were also incubated in dilute TSB. However, the bacterial direct counts would be influenced by both lytic phage infection and prophage induction, leading to an artificially low bacterial direct count and inflated IF value. It is not possible from our data to determine what effect, if any, lytic phage production may have had on our determination of IF in the bead communities. This phenomenon has been previously reported in the Gulf of Mexico, although the authors could not exclude a potential role of nutrient stimulation in prophage induction (38). Second, there may be some bias in our values, as the Bio-Sep beads most likely contain only a subset of the total bacterial community at the sampling sites, especially for beads used to trap atrazine-degrading bacteria. However, although the bead communities may not reflect an unbiased sample of the total microbial community, it is unlikely that the beads preferentially selected for predominantly lysogenic bacteria in favor of uninfected bacteria. The high IF values most likely reflect a high proportion of live and active bacteria in the beads able to respond quickly to TSB, whereas comparable assays with whole-soil communities likely include a high percentage of dead and inactive bacteria in the counts, lowering the overall IF value. Though our experiments differed somewhat from previously published aquatic studies, it is clear from our results that lysogeny is very prevalent among bacteria in agricultural soils.

Detection of pesticide-catabolic genes in phage DNA. To date, three enzymes encoded by the genes *atzA*, *trzN*, and *trzA* have been identified in soil bacteria that catalyze the dechlorination of atrazine. Based on the bacteria that have been surveyed, *atzA* appears to be found strictly in gram-negative bacteria that metabolize atrazine while *trzN* and *trzA* have been detected only in high-G+C gram-positive bacteria such as *Arthrobacter*, *Nocardioides*, and *Rhodococcus* strains. A number of gram-negative bacteria distributed among the *Alpha-*, *Beta-*, and *Gammaproteobacteria* have been isolated from geographically distinct soils, and all of these bacteria possess one or more atrazine-catabolic genes with sequences virtually identical to those of *Pseudomonas* sp. strain ADP (9). Based on these studies it has been suggested that horizontal gene transfer (HGT), presumably by conjugation, has played a significant role in the evolution of this pathway in atrazine-degrading bacteria. In the present study, the chlorohydrolase gene *trzN* was readily detected in the viral DNA fractions of induced and uninduced samples from both water- and atrazine-equilibrated beads. Interestingly, *atzA* was not detected in any of the samples examined despite its apparent widespread distribution among gram-negative atrazine-degrading bacteria. While these results do not definitively demonstrate the lateral transfer of *trzN* among soil bacteria, its presence in the viral DNA fraction, particularly in the MC-induced communities, suggests that transduction may play a role in the dissemination of this important chlorohydrolase gene in soil bacteria. It is unclear why we failed to detect *atzA* in the viral DNA of communities known to contain atrazine-degrading bacteria. Our census of lysogenic host bacteria based on detection and sequence analysis of 16S rRNA genes in the viral DNA fraction of MC-induced Bio-Sep bead communities suggests that the predominant lysogenic host bacteria (approximately 80% of clones) fell within the phylum *Actinobacteria*. As indicated previously, *atzA* has not been reported in high-G+C gram-positive bacteria; thus, if the beads were selectively colonized by actinobacteria, this may explain why *atzA* was not detected in the viral DNA fraction. Alternatively, *atzA* may simply not be subject to lateral transfer via transduction; however, given the random nature with which generalized transducing phage insert within the host genome and package DNA, this explanation also seems unlikely. Unfortunately we did not analyze the corresponding soil in which the beads were buried for *atzA* and *trzN*;

thus, it is not possible to determine if the use of Bio-Sep beads resulted in a biased sampling of the microbiota at these sites.

Horizontal transfer of 16S rRNA genes. To further assess lysogenic interactions in soil, we applied a modified method of Sander and Schmieger (23) to determine the predominant lysogenic host bacteria in the samples by constructing and analyzing 16S rRNA gene clone libraries prepared from the purified viral DNA fraction from MC-induced Bio-Sep bead communities. Our analysis revealed that the majority of cloned sequences were most closely affiliated with various genera in the *Actinobacteria* in both MC-induced water- and atrazine-equilibrated Bio-Sep beads. The predominance of *Actinobacteria* in the clone libraries may indicate that this phylum is more likely to contain MC-inducible prophage rather than biased enrichment of *Actinobacteria* in the beads. Indeed, in other similar experiments at other locations, analyses of community structure in the beads (cultivation, fluorescent in situ hybridization, and denaturing gradient gel electrophoresis) did not suggest that Bio-Sep beads selectively enriched for *Actinobacteria* (data not shown). These results are only preliminary, however, since an insufficient number of clones were analyzed to accurately assess the overall diversity of lysogenic bacteria in the samples.

16S rRNA gene sequences have of course become the gold standard for phylogenetic analyses of bacteria and bacterial communities. An inherent assumption in this approach, as pointed out by Beumer and Robinson (4), is that HGT of these genes does not occur or occurs at very low frequencies. In their recent paper, these authors demonstrated that broad-host-range generalized transducing phage could carry full-length 16S rRNA genes by sequentially passing the phage through multiple host bacteria. In all cases, 16S rRNA genes could be readily amplified from the generalized transducing phage lysates, and restriction fragment length polymorphism analysis revealed that only the 16S rRNA gene of the last host used to cultivate the phage was detected. 16S rRNA genes were not detected when the experiment was conducted with specialized transducing phage. These results are fully consistent with those of Sander and Schmieger (23), who detected 16S rRNA genes in viruses extracted from municipal wastewater, and implicate transduction as a possible mechanism for the horizontal transfer of 16S rRNA genes among bacteria.

An obvious concern with these studies as well as the results presented here involves the potential for contamination by bacteria or extracellular DNA in the purified phage lysate DNA used as template for PCR amplification of 16S rRNA genes. In this study, as in those cited above, great care was taken to prevent DNA contamination. Phage lysates were filtered through 0.22-mm filters to remove bacteria and subsequently treated with DNase to digest free DNA in the samples. Template-free PCR control reactions were included to insure that the *Taq* polymerase used was free of DNA contamination. In addition, the present study examined the purified phage extracts by EFM and transmission electron microscopy. Bacteria were not observed in either case. Finally, phage extracts were passed through Amicon spin filters to concentrate phage particles and to further remove any undigested extracellular DNA. The captured filtrate was used as a template for PCR with negative results, suggesting that DNA digestion with DNase was complete and the samples were free of contaminating DNA. Control experiments with DNA-spiked viral concentrates confirmed these results. Thus, we have a high degree of confidence that the data presented did not result from an artifact of prokaryotic DNA contamination. However, we cannot definitively exclude the possibility that nanobacteria or ultramicrobacteria may have escaped our purification procedures and affected the detection of 16S rRNA genes in the viral DNA fraction. We consider this possibility highly unlikely given the relatively narrow distribution of resulting sequences to the *Actinobacteria*. We are not aware of any studies that have definitively shown an abundance of nano-*Actinobacteria* in agricultural soils.

Heterogeneity in 16S rRNA gene sequences. Some of the 16S rRNA gene sequences amplified from viral DNA fractions (AIB-1, -11, -14, -21, and -24) showed heterogeneity in their structures. The existence of heterogeneous and mosaic 16S rRNA genes composed of intergenus (20, 27–29, 40) and even interdivision (19) sequences has already been reported in bacteria. The mechanisms accounting for the observed heterogeneity in 16S rRNA genes include misincorporation of nucleotides into DNA during replication and HGT. The possibility of putative chimera formation during PCR amplification was minimized by careful optimization of PCR conditions. We used a very high annealing temperature (58°C) and low template concentrations (approximately 50 pg DNA μl^{-1}; 50-μl reaction mixtures), which should limit the potential for chimera formation. The sequences were also rigorously checked with the program Bellerophon (12) and the powerful detection tool CCODE (11), and no chimeras were detected. By these criteria, our data represented by clone AIB-1 show a potential HGT origin of helix 43 (V7) in *Marmoricola* from the members of the family *Streptosporangiaceae* (Fig. 4). Different members of *Actinobacteria* have already been reported to have HGT origins for specific regions in their 16S rRNA molecules (27, 29, 40).

In conclusion, the high IF estimated in our study suggests that lysogenic bacteria may be more prevalent in soil than in other ecosystems. Additionally the detection of *trzN* in soil viral DNA suggests that phages may be involved in the HGT of atrazine chlorohydrolase genes among high-G+C gram-positive bacteria. HGT has been implicated as a potential mechanism to explain observed heterogeneity in 16S rRNA sequences (19, 20, 27–29, 40). Furthermore the model experiments of Beumer and Robinson (4) with broad-host-range generalized transducing phage suggest that transduction may play a role in this process. The results presented here corroborate the findings of Beumer and Robinson (4) and illustrate the potential role of transduction in horizontal transfer of 16S rRNA genes at the microbial community level. However, it is not currently possible with our data to determine the frequency of these events in soil.

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